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Specific reg II gene overexpression in the non-obese diabetic mouse pancreas during active diabetogenesis

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Abstract The reg gene, previously described in islets of 90% pancreatectomized and nicotinamide-treated rats, has been shown to be expressed in many pharmacological or surgical animal models of beta cell regeneration. We have studied the non-obese diabetic (NOD) mouse, which represents a good model of spontaneous autoimmune diabetes in which regenerative processes have recently been demonstrated. Two reg genes have been described in the mouse genome, both recognized by the human reg cDNA. In a previous work, using the human probe, we have demonstrated a strong correlation between pancreatic reg gene expression and the likelihood of developing diabetes. In the present study, we have examined the respective levels of both mouse reg I and reg II mRNA in the NOD mouse pancreas using their specific cDNA probes. We found that reg II expression was specifically prevalent compared to reg I, irrespective of sex or state of the disease. Reg II mRNA was particularly increased in overtly diabetic female mice and in cyclophosphamide-treated male mice. These data underline the need to study separately the reg genes using specific probes and show that both reg genes are subjected to various regulations, strongly suggesting that their physiological functions may be different.

Key words: Reg I; Reg II; Insulin; NOD mouse; Pancreas; Autoimmune diabetes mellitus; Pancreatic regeneration

1. Introduction

The pathogenesis of type I or insulin-dependent diabetes mellitus is characterized by immune-mediated destruction of beta cells in the islets of Langerhans of the pancreas. Some regenerating islets have been found in the pancreas of recently diagnosed diabetic patients [1]. Thus the balance between the speed of destruction and regeneration may determine the outcome of the process, i.e. overt disease or subclinical islet inflammation [2]. The non-obese diabetic (NOD) mouse provides a good experimental model of human type I diabetes [3] and O'Reilly et al. have recently provided evidence of islet neogenesis from duct epithelium in diabetic NOD mice [4]. Therefore the NOD model appears to be a valuable model for the study of the balance between destruction and regeneration of pancreatic islets. One hypothesis is that the reg gene, previously described in regenerating islets of partially pancreatectomized rats [5], could play a central role in the induction of a replicative process. In a previous study, using a human reg cDNA probe, we showed for the first time in the NOD model a strong correlation between reg gene expression and diabetic risk [6]. Indeed we found pancreatic overexpression of the reg gene in NOD female mice which are known to be prone to develop diabetes early in life whereas NOD males, which are relatively protected, have a low reg mRNA level similar to that found in a control mouse strain. The specificity of the phenomenon was reinforced by the reg overexpression observed in NOD male mice after cyclophosphamide treatment, a potent inducer of diabetes, whereas this drug had no effect in control male mice. Since two reg genes have been characterized in the mouse genome [7], these first data could not reflect the relative expression of both reg genes. In the present study, using reg I and reg II specific cDNA probes, we quantified reg I and reg II mRNA levels at various stages of diabetogenesis. We demonstrate here that the expression of the reg II gene was in all cases higher than that of reg I in the NOD mouse pancreas, for female, male and overtly diabetic female mice.

2. Materials and methods

2.1. Animals and treatment

NOD mice bred from a parental stock provided by Dr. C. Boitard (Department of Immunology, INSERM U25, Necker Hospital, Paris, France) were fed ad libitum. In our colony the cumulative prevalence of diabetes at day 210 was 43% in females and $<1\%$ in male mice. Glycosuria was detected using labsticks (Ketodiastix, Ames-Bayer Diagnostica, France).

In order to select situations with various degrees of activity of diabetogenic process we studied: (1) 22 non-diabetic NOD male mice (age range: 57–268 months); (2) 16 non-diabetic NOD female mice (age range: 53–319 months); (3) 13 overtly diabetic NOD female mice (age range: 80–248 months). The animals were killed less than 5 days after the diagnosis of diabetes; (4) six cyclophosphamide-injected NOD male mice (age 110 days). Cyclophosphamide (Endoxan, Lucien Laboratory, France) in saline was injected subcutaneously at a dose of 300 mg/kg body weight 100 days after birth. Since in our colony the percentage of overt diabetes in NOD male mice was 16% after one injection and 83% after two injections of cyclophosphamide, the animals were killed 10 days after the first injection whilst in a 'prediabetic' period.

In addition nine female and 11 male 100 day old mice (IOPS-OF1) supplied by Iffa Credo (France) were studied as controls. Six IOPS-OF1 male mice were injected with cyclophosphamide with the same protocol as described for NOD male mice.

2.2. Probes

The reg I and reg II probes were kindly provided by H. Okamoto; they were full-length cDNA, 750 bp for reg I and 700 bp for reg II, subcloned into pBluescript vector at the *Xho*I site in the polycloning site. Before hybridization, both were digested with *Xho*I enzyme and purified on agarose before labeling. The 28S cDNA probe (ATCC) was used as a control for RNA concentrations.

The insulin cDNA probe was synthesized by RT-PCR with 1 μg of total mouse pancreatic RNA according to the instructions provided

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; NOD, non-obese diabetic; IFNγ, interferon γ; OD, optical density; A.U., arbitrary units

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with the GeneAmp RT-PCR kit (Perkin Elmer Cetus). The primers used in the PCR reactions were deduced from the nucleotide sequences of mouse insulin II [8]. They were as follows: sense primer 5′-CCCACCCAGGCTTTTGTCAA-3′ (nucleotides 1193–1213) and antisense 5′-CTCCAGCTGGTAGAGGGAGC-3′ (nucleotides 1919–1938).

Thirty-five cycles of amplification were performed using the following conditions: (1) denaturation for 1 min at 95°C, (2) primer annealing for 1 min at 67°C, and (3) extension for 1 min at 72°C. After amplification, the RT-PCR products were analyzed by electrophoresis on a 10% acrylamide gel. The correct size of the fragment (258 bp) was checked by ethidium bromide visualization. The fragment was then subjected to electrophoresis on a 1.5% agarose/TAE (50×TAE:2 M Tris-acetate, 0.05 M EDTA) gel and cut up. The DNA was separated from agarose by centrifugation using Spin X column (Costar) and the correct sequence was assessed by the dideoxy chain termination method using the Sequenase sequencing kit (U.S. Biochemical Corp., Ohio).

2.3. RNA extraction and Northern blot analysis

The isolation of total pancreatic RNA was performed using the RNAzol method (Bioprobe Systems, France) according to the method described by Chomczynski et al. [9] which is particularly suitable for small samples (weight ≤ 200 mg). The RNA concentration was monitored by absorbance at 260 nm (1 OD = 40 μg/ml). The integrity of total RNA was confirmed by 1% agarose/formaldehyde electrophoresis.

Northern blot analysis of electrophoretically separated pancreatic total RNA was performed to verify the hybridization specificity of each probe. In brief, total RNA was transferred to a nitrocellulose membrane (Schleicher and Schüll, Keene, NH) and hybridized according to the procedure described by Maniatis et al. [10] with the cDNA probes labelled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham, France) using a random primer kit (Appligène, France) for reg I, reg II and insulin or a nick translation kit (Gibco BRL, Life Technologies, France) for the 28S cDNA probe. The specific activities of the probes were routinely 10^9 and 10^8 cpm/μg for random priming and nick translation labeling, respectively. Results were visualized by autoradiography.

2.4. Specificity of reg I and reg II cDNA probes

The reg I and reg II cDNA were subcloned into pBluescript, which carries bacteriophage T3 and T7 promoters, and were then transcribed, after linearization, into the sense orientation respectively by T7 or T3 RNA polymerase. After precipitation, the synthetic RNAs were subjected to electrophoresis on an agarose/formaldehyde gel and transferred on nitrocellulose [10]. Filters were hybridized with labelled reg I or reg II cDNA probes. Results were visualized by autoradiography.

2.5. Quantitative analysis of mRNA by dot blot hybridization

Sequential dilutions of total RNA were spotted on nitrocellulose membranes using a manifold apparatus (Minifold I, Schleicher and Schüll). For hybridization with reg I, reg II and insulin probes, the dilutions were from 5 μg to 0.156 μg. For hybridization with the 28S cDNA probe, we decreased the amount of total RNA 100-fold, and therefore, the sequential dilutions were from 50 ng to 1.56 ng. After spotting, the filters were baked for 2 h at 80°C before hybridization. In brief, filters were prehybridized for 4 h at 42°C in a solution containing 50% formamide, 5×SSPE (20×SSPE: 3 M NaCl, 0.02 M EDTA and 0.2 M $NaH_2PO_4 \cdot 2H_2O$), 5×Denhardt's reagent and 400 μg/ml of salmon sperm DNA. Hybridization was performed for 36 h at 42°C in the above solution containing 0.1% SDS, using 1×Denhardt's reagent instead of 5× and containing the cDNA probe labelled with [α-^{32}P]dCTP. After hybridization, filters were washed four times for 5 min in 2×SSC, 0.1% SDS at room temperature and twice for 15 min in 0.1×SSC, 0.1% SDS at 52°C. Results were visualized by autoradiography. Filter-bound radioactivity was determined by scanning autoradiograms at 490 nm using an automatic microplate reader (MR 5000 Dynatech). The mRNA concentrations were calculated from the linear regression of dot scans. For each sample, the densitometrically scanned readings for reg were corrected to the 28S signal to eliminate possible differences in total RNA loading. Results were calculated as the ratio reg I/28S, reg II/28S or insulin/28S and are expressed in arbitrary units (A.U.).

2.6. Statistical analysis

Results were expressed as means ± S.E.M. The significance of mRNA expressions between the mouse groups was determined using Student's unpaired *t*-test.

3. Results

3.1. Specificity of reg I and reg II probes

In view of the homology which exists between reg I and reg II sequences it was important to ensure the specificity of the reg I and reg II cDNA probes. We confirmed that cross-hybridization between reg I and reg II did not occur under the hybridization conditions of high stringency that we used. We prepared synthetic RNA using recombinant pBluescript for reg I or reg II cDNA. The in vitro transcription reactions were performed using T7 or T3 RNA polymerases. These RNA were hybridized with reg I and reg II cDNA probes. As shown in Fig. 1, reg I cDNA did not hybridize with reg II RNA and reg II cDNA did not hybridize with reg I RNA.

3.2. Qualitative analysis of mRNA by Northern blot

The results of Northern blot analyses are shown in Fig. 2. Each mRNA was identified as a single band and the transcript sizes were, as expected, 0.9 kbp for reg I and reg II.

3.3. Specific overexpression of the reg II gene in the NOD pancreas

3.3.1. Non-diabetic animals. The patterns of pancreatic expression of the reg I and reg II genes during aging of non-diabetic NOD female and male mice are presented in Fig. 3. Despite the large interindividual variation of values we observed a specific and significant increase of reg II mRNA compared to reg I ($P < 0.05$ for female and $P < 0.005$ for male mice). As shown in Fig. 4A, the comparison of the expression of the two reg genes before 130 days of age shows a prevalent reg II mRNA level in both female (4.97 ± 1.34 A.U. vs. 2.39 ± 0.4 A.U., $P < 0.05$) and male (3.41 ± 0.79 A.U. vs. 1.55 ± 0.27 A.U., $P < 0.05$) NOD mice. In contrast, in control OF1 mice, studied at the same age (Fig. 4B), there was a significant fourfold lower reg II mRNA level (0.09 ± 0.02 A.U. for females and 0.16 ± 0.05 A.U. for males) compared

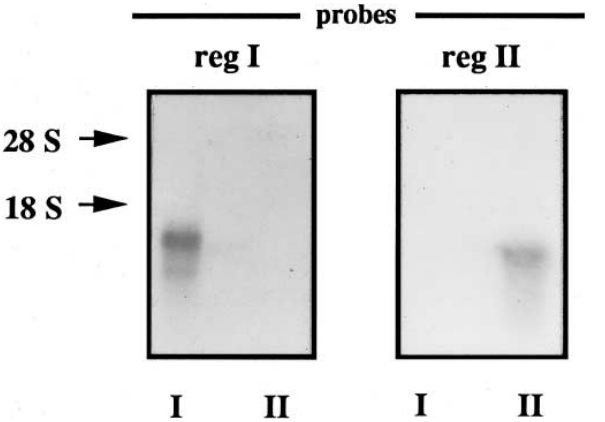

Fig. 1. Specificity of reg I and reg II probes. Synthetic RNAs from reg I and reg II cDNA, subcloned into pBluescript, were obtained after linearization using T7 or T3 RNA polymerase respectively. After electrophoresis and transfer onto nitrocellulose, the synthetic RNAs were hybridized either with a reg I cDNA-labeled probe or with a reg II cDNA-labeled probe.

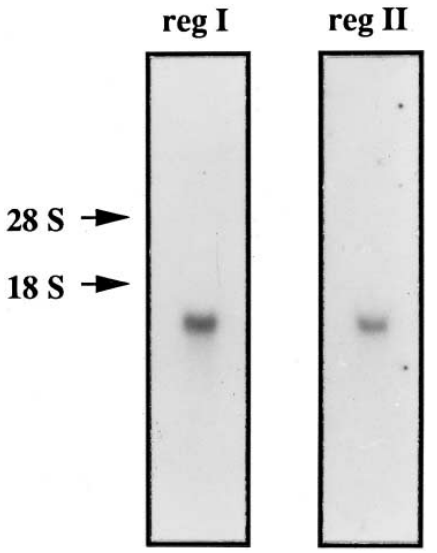

Fig. 2. Northern blot of NOD mouse pancreatic mRNA. Total RNA isolated from mouse pancreas was denatured and subjected to electrophoresis on a 1% agarose gel. After transfer onto a nitrocellulose membrane, it was hybridized with the mouse reg I cDNA-labeled probe or the mouse reg II cDNA-labeled probe. Positions of 28S and 18S rRNAs are indicated.

to reg I (0.37 ± 0.04 A.U., $P < 0.001$ for females and 0.67 ± 0.06 A.U., $P < 0.001$ for males). In addition, as previously shown with the human reg cDNA probe, the mouse reg I and reg II mRNA levels were significantly higher in NOD mice than in control OF1 mice.

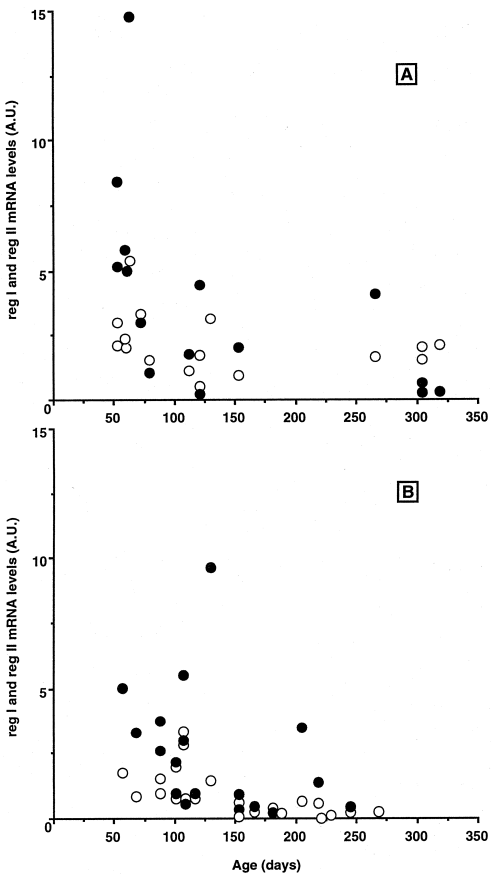

Fig. 3. Pancreatic reg I (○) and reg II (●) mRNA levels and variation with age in NOD female (A) and male (B) mice. Quantification was performed by blot as described in Section 2. Results are expressed in arbitrary units (A.U.).

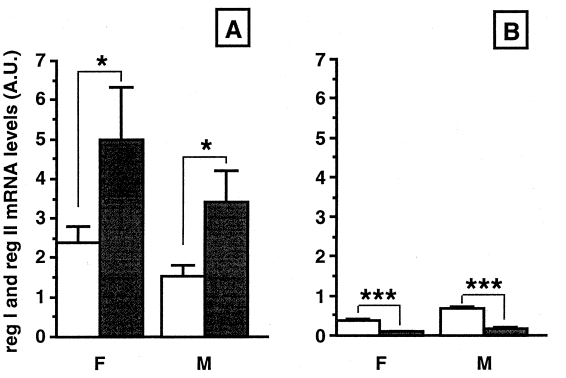

Fig. 4. Comparative levels of pancreatic reg I (□) and reg II (■) mRNA in NOD and control mice below 130 days of age. A: Diabetes-prone female and male NOD mice. B: Control female and OF1 mice. Results are expressed in A.U. as means ± S.E.M. Statistical significance indicated by $*P < 0.05$, $***P < 0.001$.

3.3.2. Diabetic female and cyclophosphamide-injected male mice. As shown in Fig. 5, the higher expression of reg II described in the NOD strain was even more evident in diabetic females with a ratio reg II/reg I of 4.07 ± 0.49 which was approximately twofold higher than the ration of the non-diabetic NOD female mice (1.77 ± 0.37). In contrast, the level of reg I mRNA expression was equivalent in diabetic and non-diabetic females (respectively 2.07 ± 0.43 A.U. and 2.16 ± 0.29 A.U.). It is interesting to note that in cyclophosphamide-injected male mice there was a much larger overexpression of the reg II gene (21.6 ± 6.86 A.U.) compared to normal male mice ($P < 0.001$); however, the reg I gene expression level (1.38 ± 0.47 A.U.) was similar in these treated animals compared with non-treated NOD males with a low risk of developing diabetes (0.91 ± 0.19 A.U.).

3.4. Insulin gene expression in NOD and OFI pancreas

In addition to reg gene expression, we studied the expression of the insulin gene in NOD mice which is critical in diabetogenesis where the insulin-producing beta cells are irreversibly destroyed. The results are presented in Fig. 6A. The diabetic NOD females did not express insulin mRNA with the exception of one, 150 days old, which exhibited an extremely

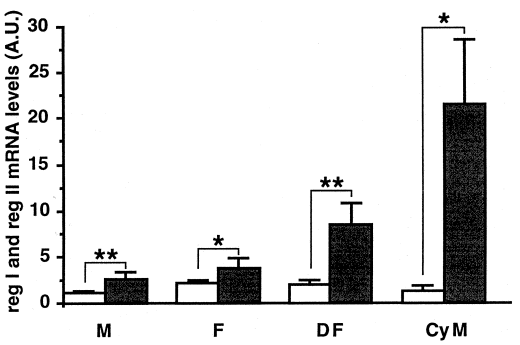

Fig. 5. Levels of pancreatic reg I (□) and reg II (■) mRNA in male (M), female (F), diabetic female (DF) and cyclophosphamide-treated male (CyM) NOD mice. Results are expressed in A.U. as means ± S.E.M. $*P < 0.05$, $**P < 0.01$.

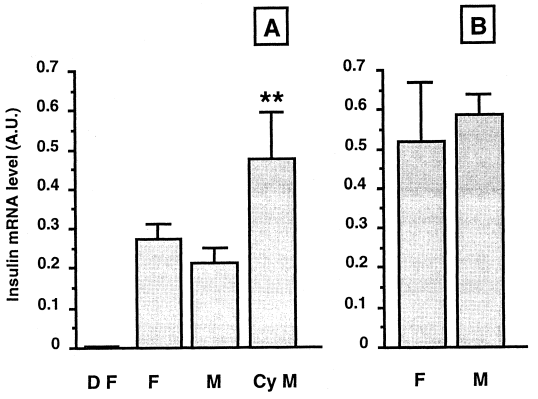

Fig. 6. Levels of insulin mRNA. A: Diabetes-prone male (M), female (F), diabetic female (DF) and cyclophosphamide-treated male (CyM) NOD mice. B: Control male (M) and female (F) OF1 mice. Results are expressed as means ± S.E.M. $^{**}P < 0.01$.

low level of insulin transcription (0.023 A.U.). No difference was found between non-diabetic NOD females and males for which the values were respectively 0.27 ± 0.03 A.U. and 0.21 ± 0.04 A.U. An increase in insulin expression (0.47 ± 0.12 A.U.) was found in cyclophosphamide-treated NOD male mice and the level was significantly higher than that measured in non-treated NOD male ($P < 0.01$) and in non-diabetic NOD female mice ($P < 0.01$).

In OF1 mice (Fig. 6B), both female and male, the insulin mRNA levels were approximately twofold higher than in NOD mice and were respectively 0.52 ± 0.15 and 0.58 ± 0.05 A.U. Finally, no increase in insulin expression was found in the OF1 cyclophosphamide-injected male mice (data not shown). In all cases no correlation was found between the expression of both reg genes and of the insulin gene.

4. Discussion

Using specific mouse reg I and reg II cDNA probes and after excluding any cross-reactivity of the probes, we demonstrate that, as observed with the human reg cDNA probe [6], there is a strong correlation between pancreatic reg gene expression and the diabetic risk. Moreover, we show that, despite the high variation in values, the expression of the reg II gene is in most cases higher than that of reg I in the NOD mouse pancreas. This phenomenon is specific to the diabetogenic strain since the relative expression of each reg gene is reversed in the diabetes-resistant strain OF1 where we found a ratio reg II/reg I of 0.24 in both male and female mice. This ratio was nearly identical to that found by Unno et al. (0.23) in the pancreas of C57BL/6J mice who were also diabetes-resistant [7]. In the NOD strain, the higher level of reg II mRNA is even more significant in overtly diabetic female mice compared to non-diabetic female mice. So in the NOD mouse, as shown in Fig. 5, there is a progressive increase in reg II expression which parallels the activity of diabetogenic processes. Indeed the reg II mRNA level was at its lowest in the resistant male, increasing in the non-diabetic female with a high risk of developing diabetes, increasing again in overtly diabetic female and finally was at its highest in the cyclophosphamide-injected male. In contrast, even if reg I gene expression is higher in the NOD pancreas compared to controls, it does not parallel the level of diabetogenicity. The small significant increase of reg I expression observed in the groups with the highest reg II expression could be explained either by a lower sensitivity to common stimulation or by the action of different stimuli. These results allow us to demonstrate that the reg gene overexpression accompanying the diabetic risk that we previously observed [6] was due to the specific overexpression of reg II. It is interesting that different behavior of the two mouse reg genes was recently reported in normal mice by Perfetti et al. [11] who demonstrated a decline in reg I but not in reg II mRNA levels during aging in normal mice. All these data underline the need to study the two reg genes separately using specific probes and show that both reg genes are subject to various regulations, strongly suggesting that their physiological functions may be different.

The meaning of reg II overexpression in NOD mice paralleling the degree of activity of diabetogenic processes remains unclear. It is tempting to consider that it is a defense mechanism against the immune-induced beta cell loss as reg has been shown to be in vitro as well as in vivo a beta cell-specific growth factor [12–14]. If this hypothesis is true, however, such a defense mechanism is largely overwhelmed in the NOD mouse as proven by the very high reg II expression found in overtly diabetic female and especially in cyclophosphamide-treated male mice. The possibility of a correlation between the diabetogenesis, a progressive increase in reg II expression and attempts at pancreatic regeneration may be tentatively evoked. It has been shown that some cytokines can upregulate the expression of reg genes [15] and many arguments suggest that the diabetogenicity of immune processes in the NOD mouse is related to a higher expression of IFNγ due to TH1-type infiltrating T lymphocytes [16]. The existence of a balance between islet destruction and regeneration and the possible central role of IFNγ in these processes are both supported by the work of Gu et al. who showed that the expression of IFNγ in the pancreatic beta cells of transgenic mice induced beta cell destruction associated with significant mitotic activity of these pancreatic cells, this latter process consisting essentially of duct cell proliferation and islet neogenesis [17]. More recently O'Reilly et al. examined for the first time the pancreatic ductal epithelium of the diabetic NOD mouse for evidence of islet regeneration [4]. They demonstrated that adult NOD mice have the capacity for duct cell proliferation and formation of ductal alpha cells and concluded that neogenesis appears to have been initiated in adult NOD mice as a result of the autoimmune process that selectively destroys pancreatic beta cells. No insulin-producing cells were observed in the duct epithelium of those animals and the authors suggest that this could be due to the absence of specific differentiating factors or autoimmune attacks.

It was therefore interesting to look for a correlation between reg I and reg II expression and the activity and/or mass of the endocrine part of the pancreas. Insulin secretion capacity is a critical point in the NOD pancreas where the insulin-producing beta cells are irreversibly destroyed by insulitis and cytotoxicity. It is admitted that pancreatic insulin content decreased regularly from approximately 6 weeks of age in NOD mice [18]. No study existed concerning the transcription rate of insulin genes. So we investigated the insulin mRNA level in NOD mice, male and female, in order to study the beta cells' capacity of transcription. In both sexes we found similar insulin mRNA levels that were approximately twofold lower than those found in the control OF1 strain. In

diabetic females (12 out of 13) no expression of the insulin gene was detected. The individual levels did not show any evident decrease in insulin gene expression with age in males or in non-diabetic females. We assumed that, despite insulitis, some female mice were able to resist diabetes for a long period and continued to express insulin genes at a relatively high rate. It is noteworthy that cyclophosphamide-injected animals exhibited higher insulin expression. This finding can be related to the hyperactivity of beta cells in the preclinical phases of the disease as suggested by others who described an increased fasting proinsulin level which preceded abnormalities of insulin secretion in twins of IDDM patients [19].

The observation of high expression of the reg II gene in NOD diabetic animals where no insulin mRNA was detectable and where regenerating endocrine cells appear to be exclusively composed of progenitor and glucagon-secreting cells led us to hypothesize that the overexpression of reg genes does not depend upon the presence of beta cells but may be associated with a regenerative process.

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